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De Novo Design of Potent Antimicrobial Peptides

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Lipopolysaccharide (LPS), shed by gram-negative bacteria during infection and antimicrobial therapy, may lead to lethal endotoxic shock syndrome. A rational design strategy based on the presumed mechanism of antibacterial effect was adopted to design cationic antimicrobial peptides capable of binding to LPS through tandemly repeated sequences of alternating cationic and nonpolar residues. The peptides were designed to achieve enhanced antimicrobial potency due to initial bacterial membrane binding with a reduced risk of endotoxic shock. The peptides designed displayed binding affinities to LPS and lipid A (LA) in the low micromolar range and by molecular modeling were predicted to form amphipathic β-hairpin-like structures when they bind to LPS or LA. They also exhibited strong effects against gram-negative bacteria, with MICs in the nanomolar range, and low cytotoxic and hemolytic activities at concentrations significantly exceeding their MICs. Quantitative structure-activity relationship (QSAR) analysis of peptide sequences and their antimicrobial, cytotoxic, and hemolytic activities revealed that site-directed substitutions of residues in the hydrophobic face of the amphipathic peptides with less lipophilic residues selectively decrease the hemolytic effect without significantly affecting the antimicrobial or cytotoxic activity. On the other hand, the antimicrobial effect can be enhanced by substitutions in the polar face with more polar residues, which increase the amphipathicity of the peptide. On the basis of the QSARs, new analogs that have strong antimicrobial effects but that lack hemolytic activity can be proposed. The findings highlight the importance of peptide amphipathicity and allow a rational method that can be used to dissociate the antimicrobial and hemolytic effects of cationic peptides, which have potent antimicrobial properties, to be proposed.

Nosocomial infections have drawn much attention from the medical and scientific communities. Several factors have posed tremendous burdens on the clinical management of such infections: (i) the ubiquity of gram-negative bacteria and the ease of acquisition of infections caused by gram-negative bacteria, (ii) the trend toward increasing numbers of antibiotic-resistant strains of gram-negative bacteria, and (iii) the lack of lasting, effective antibiotics. Therefore, the development of new and more effective antibiotics which use novel antimicrobial mechanisms of action is urgently needed. Endotoxin (or lipopolysaccharide [LPS]), a constitutive component of the outer membrane of gram-negative bacteria, is shed during infection and antimicrobial therapy and/or when the bacteria lyse (35). The resulting endotoxemia is among the leading causes of death in the developed world (22). Consequently, neutralization of LPS or its endotoxic membrane anchor moiety, lipid A (LA) (31), by a novel class of antimicrobial peptides may help to eliminate the risk of development of endotoxic shock during or after treatment of bacterial infections (6). Earlier studies on peptides derived from putative LPS-binding sequences of endotoxin-binding host defense proteins indicated that an LPS- and LA-binding motif may be formed by amphipathic sequences rich in cationic residues (9, 24). Recently, the concept of eradication of gram-negative bacteria by targeted disruption of LPS by cationic peptides was introduced (1, 6, 7). It has been proposed that even relatively short symmetric amphipathic peptide sequences containing cationic residues, such as *HBHPHBH* and *HBHBHBH* (where *B* is a

cationic residue, *H* is a hydrophobic residue, and *P* is a polar residue), with a β-sheet conformation will bind to the bisphosphorylated glucosamine disaccharide head group of LA, primarily by ion-pair formation between anionic phosphates of LA and the cationic side chains (5).

Antimicrobial peptides represent elements of innate and induced immune defenses against invading pathogens (1, 7, 20). They fold into a variety of secondary structures such as α-helical, β-sheet, cyclic, and hairpin loop peptides with one or more disulfide bridges and include the magainins, cecropins, defensins, lactoferricins, tachyplesins, protegrins, thanatin, and others (1, 2). Despite their diversity, most antimicrobial peptides share common features that include a net positive charge and an amphipathic character, which segregates hydrophilic and hydrophobic residues to opposite faces of the molecule (7, 20). Thus, antimicrobial peptides probably also share common mechanisms of bactericidal action. Although the precise mode of their action is not fully understood, it has been proposed that they target the bacterial membrane (1, 11, 20). The cationic peptides initially bind to the negatively charged LPS or LA of gram-negative bacteria (1, 2, 7, 11, 20). This initial binding leads to membrane permeation through (i) minor disruption of the phospholipid chain order and packing in the outer membrane, the "self-promoted uptake" (7); (ii) transmembrane channel formation via a "barrel-stave" or toroidal pore mechanism (10); or (iii) membrane destruction via a carpet-like mechanism (20), which may ultimately result in the killing of the bacteria. Evidence has accumulated to suggest that aggregation of amphipathic peptides on the bacterial membrane surface may be important for their antimicrobial activities (11, 20). Therefore, the design of novel antimicrobial

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Ac-<u>C-VKVKVKV-GSG-VKVKVKV-C</u>-*NH₂*
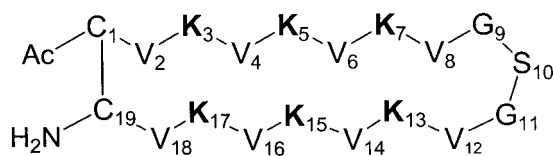
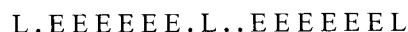L . E E E E E E . L . . E E E E E E L

FIG. 1. Sequence and chemical structure of designed cyclic cationic peptide V1. The V peptides are composed of two symmetric amphipathic LPS- and LA-binding motifs, *HBHB(P)HBH* (see Table 1 for the sequences), that form two strands of a β-hairpin joined by a $G_9S_{10}G_{11}$ turn on one side and a disulfide bond between C_1 and C_{19} bridging the N- and C-terminal residues on the other side. Prediction of the secondary structure preferences of the V-peptide sequences within a protein environment was done with the PHD program (25). E, extended (β sheet); L, loop structure; dots, random conformation.

peptides can be based on the similarities between the endotoxin-binding and antimicrobial cationic peptides, since both these effects require similar structural features of the peptides, namely, cationic and amphipathic characters. It can be expected that a strong interaction of the peptides with LPS or LA will promote their destructive action against the bacterial membrane and reduce the risk of development of endotoxemia. In fact, potent cyclic antimicrobial peptides selective for gram-negative bacteria on the basis of the β-stranded framework mimicking the putative LPS-binding sites of the LPS-binding protein family have been successfully developed (19).

Certain antimicrobial peptides show affinities not only to bacteria but also to higher eukaryotic cells, even though the outer leaflet of healthy mammalian cells is composed predominantly of neutral (zwitterionic) phospholipids (11, 20). Structure-activity studies with these antibacterial peptides indicate that changes in the amphipathicity could be used to dissociate the antimicrobial activity from the hemolytic activity (13). Recently, it was shown that peptide cyclization increases the selectivity for bacteria by substantially reducing the hemolytic activity (21).

We report here on the de novo design of synthetic cationic peptides with two LPS- and LA-binding sites which show structural similarity to cyclic β-sheet defense peptides, such as protegrin 1, thanatin, and androctonin (2). Systematic modifications of molecular properties made by varying the amino acid residues of the amphipathic LPS- and LA-binding motifs (5) while preserving the size, symmetry, and amphipathic character of the peptides are described. The purpose of these substitutions was to determine whether introduction of the binding affinity to endotoxin could enhance the antimicrobial activities of cyclic cationic peptides. Furthermore, we have investigated whether directed substitutions can selectively increase the antimicrobial potencies independently from the hemolytic activities of the peptides.

Seven cationic peptides, each cyclized via a disulfide bridge (Fig. 1), were synthesized and their biological properties were characterized. Quantitative structure-activity relationships (QSARs) were obtained by linking the experimental potencies to simple physicochemical molecular properties that

can be easily derived from the peptide sequences. Implications for the molecular mechanism of the antibacterial effect are suggested, and practical guidelines for the design of nonhemolytic highly active antimicrobial peptides are proposed.

MATERIALS AND METHODS

Molecular modeling. Molecular models of peptides V1 to V7 were constructed so that they formed amphipathic β-hairpin-like structures containing two identical LPS- and LA-binding sequences connected by the GSG loop, with patterns of secondary structure predicted by use of the PHD algorithm (25). A disulfide bridge between the N- and C-terminal residues (Cys_1 and Cys_{19}, respectively) stabilized the 19-residue cyclic peptides (Fig. 1). The models were built with the Insight II program (Accelrys, San Diego, Calif.). The coordinates of the *Escherichia coli* LA were taken from Frecer et al. (5). The model of the reference cationic peptide antibiotic, polymyxin B (PmB), was obtained from nuclear magnetic resonance measurements of the PmB-LA complex (23).

Molecular mechanics. Molecular mechanics (MM) simulations with the V peptides, PmB, and LA and their 1:1 complexes were carried out with the Discover program (Accelrys) by using an all-atom representation. Class II consistent force field CFF91 (16) was used. The geometries of all molecular structures were extensively minimized by using conjugate gradient minimization (5).

Structural and dynamic properties of peptides. Initial minimized models of the cyclic peptides were placed in a periodic solvent box containing approximately 700 water molecules and were subjected to molecular dynamics (MD) simulation. The system was heated from 0 to 300 K over 5 ps and was equilibrated for 5 ps. The MD simulation time step was set to 1 fs, and the integration was carried out with the Verlet algorithm. An ensemble of 100 configurations was collected over 100 ps at 1 configuration/ps for calculation of the mean values of the geometric parameters and estimation of conformational entropy after energy minimization.

Docking of LA to peptides. A flexible induced-fit docking method based on MD simulation was used to dock the LA monomer to the V peptides and PmB, which allowed for the full flexibility of the ligand and the receptor (5). The docking search produced >100 stable configurations of the peptide-LA complex for each V peptide. The configuration with the lowest total energy and the lowest peptide-LA interaction energy (negative values) was used as the starting point for the droplet model MD simulation, in which the complexes were enclosed by three solvation shells and were thermally equilibrated (5).

Estimation of binding affinity. The relative affinity of reversible binding of solvated LA to the cationic peptides to form a peptide-LA complex (the computed binding affinity [$\Delta\Delta E_{comp}$]) was estimated (5) with reference peptide PmB.

Lipophilicity and amphipathicity parameters. The peptide lipophilicity index ($\Pi_{o/w}$) of each of the V peptides was estimated as the sum of experimental lipophilicity parameters of amino acids ($\pi_{o/w}$) of all residues, defined for side-chain partitioning in the system *n*-octanol–water (3). The amphipathicity index (AI) was calculated as the sum of side-chain parameters $\pi_{o/w}$ over a subset of residues containing basic and polar amino acids [AI = $\Sigma_i^{PF}(\pi_{o/w})_i$ (residues with odd sequential numbers, *i*; Fig. 1)], which is referred to as the polar face (PF) of the cyclic V peptide. The subset containing the lipophilic *H* residues defines the nonpolar face of the V peptide (residues with even sequential numbers).

Peptide synthesis. The antimicrobial V peptides were synthesized by Genemed Synthesis (South San Francisco, Calif.) by solid-phase synthesis and standard 9-fluorenylmethoxy carbonyl chemistry and were purified to >95% purity by reverse-phase high-pressure liquid chromatography. A cysteine derivative with an acetamidomethyl protecting group was used for disulfide bridge formation. The peptide composition and the efficiency of complete cyclization were confirmed by mass spectrometry.

SPR. The kinetics of the real-time interaction of the peptides with LA from *E. coli* F-583 (Sigma, St. Louis, Mo.) were determined from the surface plasmon resonance (SPR) determined with an HPA biosensor chip (BiaCore 2000; Biacore, Uppsala, Sweden). The peptide association and dissociation rates for 100 to 500 µM solutions of the V peptides or PmB (Medipharm, Bedford, Ohio) were determined in pyrogen-free water (Baxter, Morton Grove, Ill.) by the method of Tan et al. (32).

CD spectroscopy. The circular dichroism (CD) spectra of the peptides at 40 µM were recorded at 25°C in water and in the presence of small unilamellar vesicles (3:1 ratio of phosphatidylcholine and 0.75 nM LA), as described by Tan et al. (32).

Antibacterial activity test. MIC testing was performed as a modification (40) of the method proposed by the Hancock Laboratory (MIC Determination for Cationic Antimicrobial Peptides by Modified Micro Titer Broth Dilution

TABLE 1. Molecular properties of cyclic V peptides with variable symmetric cationic LPS- and LA-binding motifs

Peptide	V-peptide sequence[a]	Q_M (charge of one electron, è)	AI[b]	$\Pi_{o/w}$[c]	$\Delta\Delta E_{comp}$ (kJ mol^{-1})[d]	K_d (μM)[e]
V1	Ac-C-*VKVKVKV*-GSG-V*KVKVKV*-C-NH$_2$	6	−2.86	6.86	−33	0.8
V2	Ac-C-*VKVSVKV*-GSG-*VKVSVKV*-C-NH$_2$	4	−0.96	8.76	192	2.1
V3	Ac-C-*VKVRVKV*-GSG-V*KVRVKV*-C-NH$_2$	6	−2.90	6.82	−154	1.9
V4	Ac-C-*VKVQVKV*-GSG-V*KVQVKV*-C-NH$_2$	4	−1.32	8.40	25	2.8
V5	Ac-C-*AKAKAKA*-GSG-*AKAKAKA*-C-NH$_2$	6	−2.86	−0.42	63	0.9
V6	Ac-C-*FKFKFKF*-GSG-*FKFKFKF*-C-NH$_2$	6	−2.86	11.42	−443	9.4
V7	Ac-C-*WKWKWKW*-GSG-W*KWKWKW*-C-NH$_2$	6	−2.86	15.10	−226	7.5
PmB	R-*ĐTĐĐĐFLĐĐT*	5	—[h]	—	0	0.7[g]

[a] In the cyclic peptide models (Fig. 1) the two symmetric amphipathic sequences *HBHPHBH* and *HBHBHBH* form a dual LPS- and LA-binding motif (5) and the peptides are cyclicized via a C_1-C_{19} disulfide bridge. The general sequence is Ac-C-*HBHB(P)HBH*-GSG-*HBHB(P)HBH*-C-NH$_2$.

[b] AI was defined as the sum of $\pi_{o/w}$ over a subset containing *B/P* residues (all residues with odd numbers in the sequence; Figure 1) of the cyclic peptide [AI = $\Sigma_i^{PF}(\pi_{o/w})_i$]. $\pi_{o/w}$ is defined by means of the interphase partitioning coefficients of the side chains measured in the *n*-octanol–water system (3).

[c] The $\Pi_{o/w}$ of the peptides was derived as the sum of lipophilicity parameters $\pi_{o/w}$ over all residues in the peptide as $\Pi_{o/w} = \Sigma_i^{PF}(\pi_{o/w})_i$.

[d] $\Delta\Delta E_{comp}$ of peptide-LA complex formation from MD simulations with solvated peptides [(Pep$_i$)$_{aq}$] and LA [(LA)$_{aq}$] and the peptide-LA complex (where aq represents aqueous) was computed [(Pep$_i$-LA)$_{aq}$] as $\Delta\Delta E_{comp} = \Delta\Delta E_{MM} + \Delta\Delta E_{solv}$, where $\Delta\Delta E_{MM}$ is the relative MM interaction energy contribution to $\Delta\Delta E_{comp}$, and $\Delta\Delta E_{solv}$ is the relative solvation energy contribution (by using PmB as the reference peptide): $\Delta\Delta E_{MM} = [<E_{MM}(Pep_i-LA)> - <E_{MM}(Pep_i)> - <E_{MM}(LA)>] - \Delta E_{MM}$ (PmB-LA) and $\Delta\Delta E_{solv} = [<E_{solv}(Pep_i-LA)> - <E_{solv}(Pep_i)> - <E_{solv}(LA)>] - \Delta E_{solv}$ (PmB-LA), where solv is solvation and where $<E_{MM}>$ and $<E_{solv}>$ are averaged energies for the ensemble; entropic contributions were considered constant for all V peptides.

[e] The K_d values for the peptide-LA complex obtained from association (k_1) and dissociation (k_{-1}) rate constants were computed as k_{-1}/k_1. The rate constants were determined from SPR experiments with an *E. coli* LA monolayer by using an HPA biosensor chip at 25°C on a BiaCore 2000 instrument (Fig. 5).

[f] The sequence of the reference antibiotic, PmB, from *Bacillus polymyxa*, a cyclic cationic LPS- and LA-binding peptide; Đ, α,γ-diaminobutyric acid (Dab); Đ, the neutral form of α,γ-diaminobutyric acid with the side chain amino group attached to the backbone of the C-terminal Thr; Phe, the D enantiomer; R, is an aliphatic chain, CH$_3$(CHCH$_3$)(CH$_2$)$_4$CO—.

[g] Published K_d values of PmB bound to LA from *E. coli*, measured by titration calorimetric studies and fluorescent probe displacement experiments at 25°C, range from 74 to 370 nM (27, 30). These K_d values are about 2 to 10 times lower than the K_d value of 700 nM from SPR measurements carried out in our laboratories.

[h] —, not applicable.

Method). Briefly, 100 μl of 2×10^5 to 7×10^5 CFU of bacterial suspension per ml of Mueller-Hinton broth (Becton Dickinson, Sparks, Md.) was dispensed into sterile 0.2-ml polypropylene tubes. Eleven microliters of serially diluted peptides in 0.01% acetic acid and 0.2% bovine serum albumin (Sigma) over a concentration range of 4 to 692 nM (0.01 to 1.25 μg/ml) was then added. The cultures were shaken at 37°C for 18 to 24 h. Viable cell counts were determined by the standard drop-count method (36). Cultures without the peptides were used as positive controls. Uninoculated Mueller-Hinton broth was used as a negative control. The tests were carried out in triplicate.

Hemolytic activity assay. Hemolytic activity was measured as the amount of hemoglobin released by lysis of human erythrocytes incubated for 1 h with serial twofold dilutions of the V peptides at concentrations ranging from 0.01 to 375 μg/ml in pyrogen-free saline at 37°C (28).

Cytotoxicity test. The cytotoxic activities of the V peptides were tested by measuring the bioreduction of Cell Titer 96 AQueous One Solution Reagent (Promega, Madison, Wis.) by healthy THP-1 human monocytes (2×10^4 cells) incubated for 1 h at 37°C with serial twofold dilutions of the V peptides at concentrations ranging from 0.01 to 400 μg/ml in pyrogen-free saline (32).

Competitive inhibition of endotoxin induction by *Limulus* amebocyte lysate (LAL) assay. The efficiencies of the V peptides to bind to LPS in solution was measured with a Kinetic-QCL kit (BioWhittaker, Walkersville, Md.) at 37°C in the presence of 5 endotoxin units (EU) of LPS per ml and V peptides at concentrations ranging from 0.1 to 3.9 μM (32). The LPS-binding curves were used to determine the K_ds of the peptide-LPS complexes and the number of LPS molecules that bound to a single V-peptide moiety.

RESULTS

Design of peptides. We have designed a series of cyclic cationic peptides for which a high affinity of binding to LA was predicted from molecular modeling. The peptides were composed of two identical symmetric amphipathic LPS- and LA-binding motifs containing seven alternating *H* and *B* or *P* residues with the general sequence Ac-C-*HBHB(P)HBH*-GSG-*HBHB(P)HBH*-C-NH$_2$ (Fig. 1), where Ac is an acetyl group. The actual sequences of the binding motifs are given in Table 1. Each cyclic peptide included a GSG loop sequence,

which enabled enhancement of the LPS- and LA-binding affinities of the tandemly repeated endotoxin-binding motifs, and a Cys$_1$-Cys$_{19}$ disulfide bridge linking the terminal residues. To achieve a high level of antimicrobial activity and selectivity toward bacteria instead of eukaryotic cells, the peptides were optimized by adjusting their molecular properties. In peptides V1 to V7, the molecular charges, amphipathicities, and lipophilicities of the peptides have been modulated by varying the cationic (polar) amino acid residues in the center of the binding motifs, where *B*(*P*) is Lys or Arg(Ser or Gln), and the hydrophobic residues, where *H* is Ala, Val, Phe, or Trp, which preserved the symmetries, sizes, and amphipathic characters of the peptides with alternating polar and nonpolar residues (Table 1). Lysine residues were previously shown to contribute the most toward the high affinity to LA when they were placed at the flanking basic residue position of the *H***B**H**B**(*P*)*H***B**H motif with a β-sheet conformation (5).

Computational prediction of peptide flexibility. MD simulations showed that the backbone conformations of free V peptides evolved from the initial β-hairpin with defined patterns of secondary structure into flexible random conformations (data not shown). The φ and ψ backbone torsion angles of the central *B* or *P* residues of the LPS- and LA-binding motifs (residues 5 and 15), which characterize the local secondary structure, drifted to random conformations and their mean values fluctuated during the simulation, with similar high standard deviations of up to ±100°. Two interatomic distances between the C$_\alpha$ carbons of residues 5 to 15 and 1 to 10 (Fig. 1), which describe the overall shape of the peptide backbone, evolved to time-averaged values corresponding approximately to the circular shapes of the solvated peptides and fluctuated with similar standard deviations of up to ±5 Å. The patterns of
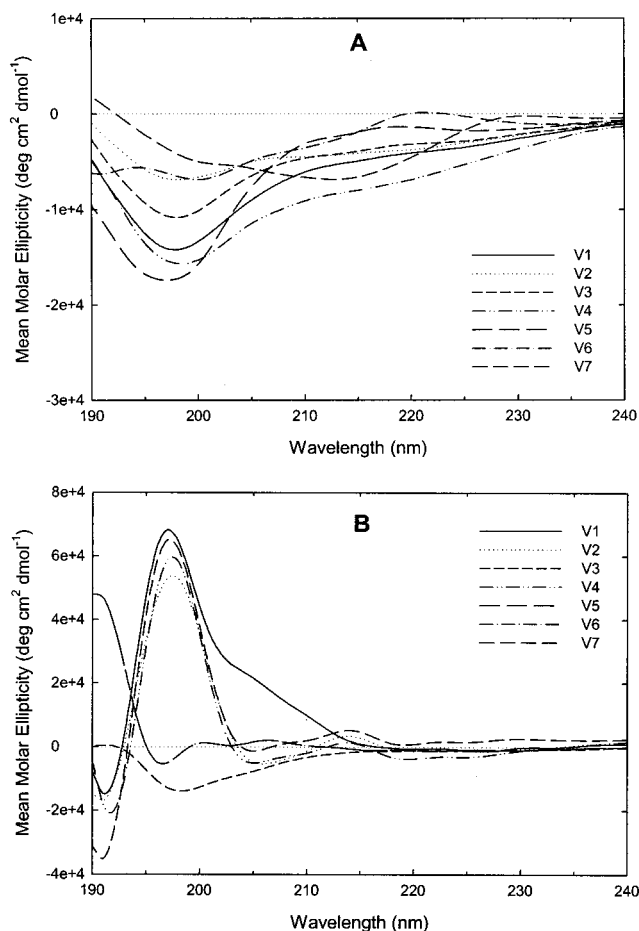
FIG. 2. CD spectra of cationic V peptides. (A) Spectra of peptides V1 to V7 recorded in water; (B) spectra of peptides V1 to V7 recorded in the presence of phosphatidylcholine and *E. coli* LA (molar ratio, 3:1), which form small lipidic vesicles that mimic the surface of a bacterial membrane.

the molecular shape fluctuations and torsional flexibility indicate high degrees of flexibility of the free V peptides in solution. Therefore, we can assume that the entropic contributions to the receptor binding affinity associated with the internal degrees of freedom of the V peptides will also be similar.

CD spectroscopy. The CD spectra provide only low-resolution information. Therefore, interpretation in terms of the V-peptide secondary structure is problematic (37), although differences between the V1 to V4 peptides and the V6 and V7 peptides, with the last two peptides containing aromatic *H* residues, are evident (Fig. 2A). Nevertheless, comparison of the CD spectra measured in water (Fig. 2A) and in the presence of small unilamellar vesicles that mimic the bacterial membrane surface (18) (Fig. 2B) indicates that the peptides underwent conformational transitions in the vicinity of the heterogeneous solvent-membrane interface. This observation is in agreement with the findings from the MD simulations, which indicated a high degree of flexibility of the free peptide backbones in water.

Mode of LPS binding to V peptides. The binding of LPS to V1 and V2, which represent peptides with two different types of binding motifs, was measured by the competitive inhibition
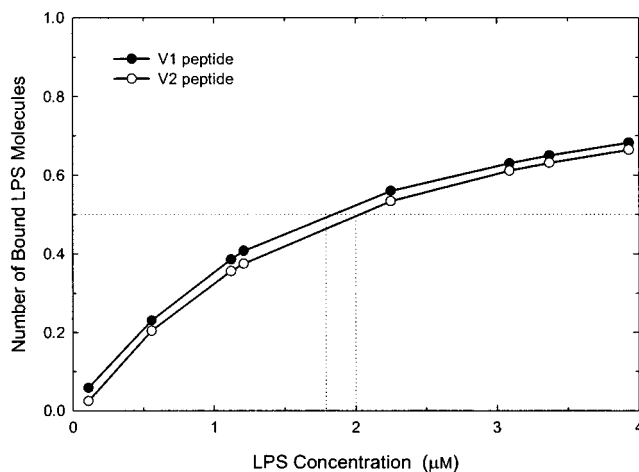

FIG. 3. Typical curves for binding of LPS to V peptides, represented by the V1 and V2 peptides. The peptide-LPS association is characterized by curves of the average number of bound LPS molecules (n) per single peptide molecule (v), where $v = n[\text{peptide-LPS}n]/([\text{LPS}] + n[\text{peptide-LPS}n])$ plotted against the ligand concentration ([LPS]). These curves allow estimation of the number of binding sites per peptide when the asymptote of v is equal to 1. The K_d of the peptide-LPSn complex was read for v equal to 0.5.

of LPS-induced binding by the LAL assay (32). The number of LPS- and LA-binding sites (n) on the V peptides during the association peptide + nLPS$\leftrightarrow$ peptide-LPS$_n$ was determined from the plot of the average number of bound LPS molecules per peptide molecule (v) as the asymptote v equal to 1 is approached by the binding curve (v versus LPS concentration) at high ligand concentrations (Fig. 3). The binding curves for V1 and V2 provided estimates of the K_d values of a 1:1 peptide-LPS complex (at v equal to 0.5) as 1.8 and 2.0 μM, respectively.

Hill's plot showed slopes of n equal to 0.995 and 1.205 for the V1 and V2 peptides, respectively, indicating the presence of a single noncooperative LPS- and LA-binding site on each
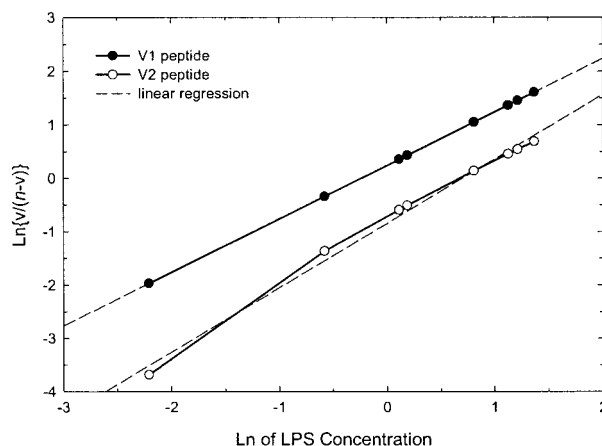

FIG. 4. Hill's plot for LPS binding to V1 and V2 peptides. The number of LPS-binding sites on each peptide molecule (Hill's coefficient, n) was derived from the slope of the plot $\ln[v/(n - v)] = \ln K_d + n\ln[\text{LPS}]$ (see legend to Fig. 3). Linear regression (dashed lines) supplied the slopes n equal to 0.995 and 1.205 for the V1 and V2 peptides, respectively, with correlation coefficients of 0.999 and 0.992, respectively (in theory, n is equal to 1 for a single binding site).
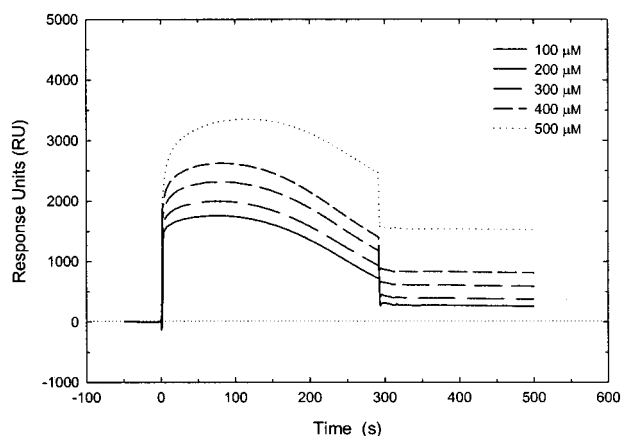
FIG. 5. Representative SPR sensorgram of V peptides. Sensorgrams indicate the association and dissociation phases of V1 peptide binding to an LA monolayer immobilized on an HPA chip. The fits of the rate constants to the association and dissociation curves yielded a K_d of 0.78 μM. The dissociation rates may be somewhat underestimated due to ligand rebinding, which is a common feature of SPR experiments (33).

peptide molecule (Fig. 4). This observation suggests that V peptides form complexes with LPS or LA with a 1:1 stoichiometry.

Affinities of binding to LA. A representative SPR sensorgram displaying the interaction of the cationic V1 peptide with the anionic LA monolayer coated on the HPA biosensor chip (Fig. 5) shows a concentration-dependent increase in response units upon peptide binding. These changes corresponded to the average rate constants of the peptide-LA association ($k_1 = 8.25 \times 10^2$ M^{-1} s^{-1}) and dissociation ($k_{-1} = 6.47 \times 10^{-4}$ s^{-1}), which define a K_d of 7.8×10^{-7} M. Thus, the cyclic V peptides exhibited micromolar and submicromolar K_ds for the peptide-LA complexes comparable to that for the reference antiendotoxin agent, PmB, a cyclic cationic peptide, which was observed to bind to the *E. coli* LA with a K_d of 7.1×10^{-7} M (Table 1) (27, 30).

Computational prediction of affinities of binding to LA. To gain more detailed structural information about the nature of the interactions of V peptides with the LA moiety, the 1:1 complexes of peptide-LA were modeled. The two identical LPS- and LA-binding sequences [*HBHB(P)HBH*] separated by the GSG turn formed a single binding site. The MD simulations have shown that the bound conformations of V1 to V7 resemble a β-hairpin-like structure induced by the proximity of the amphipathic LA moiety. The bound peptides displayed a distinct polar face, formed mainly by the side chains of *B* and *P* residues, and a nonpolar face, constituted by the *H* residues (Fig. 6). In the complexes, the cationic residues of the binding motifs formed ion pairs with the anionic phosphates of LA. The amide and ester linkages of LA formed a network of hydrogen bonds with the peptide backbone, while the fatty acid chains of LA established hydrophobic contacts with the chains of the *H* residues (Fig. 6). The relative binding affinities of V peptides to LA ($\Delta\Delta E_{comp}$; Table 1) were calculated with reference to the binding affinity of the antiendotoxin peptide PmB.

Within the homogeneous subgroup, the V1 to V4 peptides,

which contained conserved hydrophobic residues (all *H* residues were Val), peptides V2 and V4, which had lower molecular charges (4 è [è is the charge of one electron]), showed higher $\Delta\Delta E_{comp}$ values (weaker binding). On the other hand,
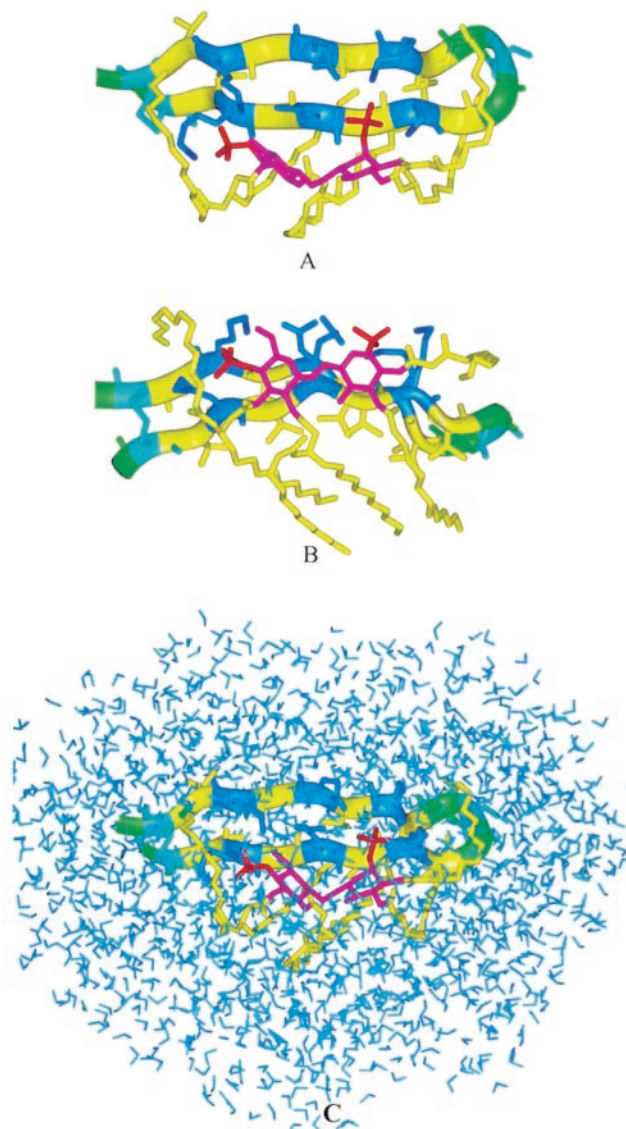

FIG. 6. Computer model of peptide-LA complex derived by MD-assisted docking in water. (A) In the model LA is attached to the dual LPS- and LA-binding sequence of peptide V4. The ribbon shows the backbone conformation of the V4 peptide (Ac-*C-VKVQVKV*-GSG-*VKVQVKV*-C-NH$_2$), which acquired a β-hairpin-like structure in the 1:1 complex with the LA monomer in water. The β-hairpin supersecondary structure of V4 was induced by the amphipathic LA counterpart. Residue side chains and LA are shown in stick representation. Hydrophobic Val residues (yellow) interact primarily with fatty acid chains of LA (yellow). Cationic Lys residues (blue) form ion pairs with the anionic phosphate groups (red) of the LA head group (purple). Hydrogen atoms were omitted for clarity. (B) Side view of the V4-LA complex. (C) The microscopic droplet discrete solvation model used in the MD-assisted docking of the LA molecule to V4 and thermal averaging of the V4-LA complex shows that it contains three layers of water molecules surrounding the complex (four solvation shells with about 2,000 H$_2$O molecules, shown in stick representation).

TABLE 2. Antimicrobial, cytotoxic, and hemolytic activities of cyclic cationic V peptides

Peptide	Antimicrobial activity (MIC [nM])[a]					Cytotoxic activity (EC$_{50}$ [nM]) for THP-1 cells	Hemolytic activity (EC$_{50}$ [nM]) for human erythrocytes	SI range[b]
	E. coli ATTC 25922	*P. aeruginosa* ATTC 27853	*Klebsiella pneumoniae* ATTC 13883	*Salmonella enterica* serovar Typhimurium ATTC 14028	*Shigella sonnei* ATTC 25931			
V1	≤5	≥619	145	≤5	≤5	45,000	880,000	≤1,420–176,000
V2	≤5	≥643	643	≤5	≤5	5,660,000	5,89,000	≤915–117,800
V3	300	≤5	10	300	≤5	42,000	590,000	1,967–118,000
V4	10	≤5	<5	≤5	10	88,000	3,226,000	46,086–645,200
V5	173	≤5	≥692	≤5	692	140,000	1,055,000	≤1,525–211,000
V6	518	≤4	≤4	518	≤4	45,000	35,000	68–8,750
V7	114	≤4	458	≤4	7	85,000	45,000	98–11,250

[a] The MIC was defined as the lowest concentration of the V peptide that fully inhibited the growth of bacteria; from the literature, the MIC for PmB *P. aeruginosa* is reported to range from 1 to 100 μg/ml (27, 36). This work determined the MIC of PmB to be 830 nM (1 μg/ml).
[b] The SI range is calculated as the ratio of concentrations causing hemolytic and antimicrobial activities and reflects the range of specificities of the peptides for the gram-negative bacteria over those for human erythrocytes.

in the V1, V5, V6, and V7 peptide subgroup, which had identical polar faces (all *B* residues were Lys) and a variable hydrophobic face (where *H* was Ala, Val, Phe, or Trp), the presence of smaller, less hydrophobic residues (Ala or Val) resulted in binding stronger than that when bulkier aromatic (Phe or Trp) residues were present.

Antimicrobial activity. The V peptides displayed a wide range of antibacterial potencies against five gram-negative bacterial species, with the concentrations at which they showed activity ranging from 4 to ≥692 nM (Table 2). Most of the peptides showed specificity for individual gram-negative bacterial species, resulting in up to 138-fold differences in the MICs of the same derivative. The MIC of the reference antimicrobial peptide, PmB, for *Pseudomonas aeruginosa* was determined to be 830 nM (1 μg/ml). Reports in the literature give a range of potencies against *P. aeruginosa* ranging from 1 to 100 μg/ml (27, 36).

It is noteworthy that the range of MICs of peptides V1 to V7 (Table 2) was up to 208-fold lower than that of reference antibiotic PmB, which has an MIC of 830 nM (1 μg/ml). The most potent peptide, V4, displayed a broad spectrum of antibacterial activity, with the lowest MIC being 4 nM (10 ng/ml) (Table 2).

Hemolytic activity. The effective concentration that caused 50% erythrocyte lysis (EC$_{50}$) exceeded the nanomolar range of the antimicrobial activity by 3 to 4 orders of magnitude (Table 2). At nanomolar concentrations, the V peptides showed no hemolytic activity. The V6 and V7 peptides, which contain aromatic Phe and Trp residues and which possess the highest experimental lipophilicity index, $\Pi_{o/w}$ (Table 1), also showed the highest degrees of hemolytic activity, while the less lipophilic V4 and V5 peptides exhibited the lowest activities. With an EC$_{50}$ of 3.2 mM (6.5 mg/ml), the hemolytic activity of the most active peptide, V4, was almost three times lower than that of the reference antibiotic, PmB (27).

The range of specificity indices (SIs), in which SI describes the specificities of the V peptides for gram-negative bacteria over that for human erythrocytes, was calculated as EC$_{50}$ for hemolysis/MIC for gram-negative bacteria. The majority of the peptides exhibited improved specificities and increased SIs compared to those for PmB (Table 2), for which the estimated SI is 190 (27). The SI of the most active peptide, V4, demon-

strated a greater than 2,400-fold increase compared to that of PmB, which resulted from the higher level of antimicrobial activity and the lower level of hemolytic activity of the V4 peptide.

Cytotoxic activity. The EC$_{50}$s of the V peptides for cytotoxicity ranged from 40 μM to 5.7 mM, which exceeds their MICs by up to 3 orders of magnitude (Table 2). The peptide with the highest SI, V4, exhibited 0% cytotoxicity toward human monocytes at nanomolar concentrations. The V4 peptide caused 50% cell lysis only at an EC$_{50}$ of 88 μM (180 μg/ml), which is about 4 orders of magnitude higher than the concentration needed for its antimicrobial effect.

QSARs. The hypothetical mechanism of bacterial membrane disruption by cationic amphipathic peptides may involve several molecular properties of the peptides that are related to the individual stages of the process: a net positive charge (attachment to anionic outer membrane constituents), amphipathicity (aggregation on the membrane surface), and lipophilicity (permeation into the membrane). It is likely that only those peptides which possess a balanced combination of these properties can achieve sufficient activity in each step of the concerted mechanism and attain higher levels of antimicrobial effects. Therefore, an analysis that compares these properties to the observed biological effects can provide valuable insight into the relationships between the sequences of antimicrobial peptides and their potencies. Simple properties which can be derived from the peptide sequences, such as the molecular charge (Q_M), AI, and $\Pi_{o/w}$, were correlated to the mean antimicrobial effect against gram-negative bacteria by multivariate linear regression:

$$\ln(\text{MIC}) = 9.49 Q_M + 10.17 \text{AI} - 0.05 \Pi_{o/w} - 22.16 \quad (1)$$

The three-parameter correlation equation showed promising statistical characteristics [$n = 7$ samples, correlation coefficient (R^2) = 0.99, leave-one-out cross-validated correlation coefficient (R_{xv}^2) = 0.98, standard error (σ) = 0.23, F-test statistic (F) = 69.85, confidence level (α) > 95%] and pointed to an important relationship. Namely, the t statistics of the correlation, which describe the contribution of each individual variable to the multivariate regression model, revealed that Q_M and AI

represent the leading terms and were strongly related to the antimicrobial activities of the V peptides.

Both the antimicrobial and the hemolytic activities of the cationic peptides involve cell membrane lysis and have been reported to depend on the same physicochemical properties (14, 21, 38). A similar multiparameter correlation equation was obtained for the hemolytic activities of the V peptides:

$$\ln(\text{EC}_{50} \text{ for hemolysis}) = -5.34Q_M - 4.94\text{AI}$$

$$- 0.23\Pi_{o/w} + 31.87 \qquad (2)$$

The correlation parameters were as follows: $n = 7$, $R^2 = 0.92$, $R_{xv}^2 = 0.84$, $\sigma = 0.76$, $F = 7.40$, and $\alpha > 95\%$. In this case the t statistics showed that the hemolytic effect was influenced primarily by molecular lipophilicity. For the cytotoxic effects of the V peptides, we obtained the correlation equation

$$\ln(\text{EC}_{50} \text{ for cytotoxicity}) = 8.98Q_M + 11.74\text{AI}$$

$$- 0.04\Pi_{o/w} - 8.70 \qquad (3)$$

The correlation parameters ($n = 7$, $R^2 = 0.98$, $R_{xv}^2 = 0.96$, $\sigma = 0.47$, $F = 27.03$, $\alpha > 95\%$) and t statistics indicated that the cytotoxic activity was determined mainly by the peptide charge and amphipathicity.

Only the combination of three molecular properties (charge, amphipathicity, and lipophilicity) was found to correlate with the observed antimicrobial, hemolytic, and cytotoxic activities of the V peptides. Single-variate QSAR correlations of these properties to the biological effects could not be established, suggesting that the membrane disruption may involve a concerted process. However, the t test of the multivariate correlation equations (equations 1 to 3) revealed that the antimicrobial effect on bacteria was determined predominantly by the V-peptide charge and amphipathicity, i.e., by the number of cationic and polar residues forming the polar face of the V peptides and their distributions throughout the two symmetric amphipathic LPS- and LA-binding motifs. On the other hand, the hemolytic activity against eukaryotic cells was influenced mainly by the molecular lipophilicity, i.e., the sum of the lipophilicities of all residues, with the major contribution coming from the H residues, which form the nonpolar face of the V peptides, which is predicted to acquire a β-hairpin-like structure in the peptide-LA complexes.

DISCUSSION

Binding affinities of V peptides to LA. The binding affinities of the V peptides computed for the LA monomer and the K_ds derived from SPR measurements with an LA monolayer represent two diverse models for the prediction of the interaction of peptides with the outer membranes of gram-negative bacteria. The first theoretical model simulates the binding of a single free LA molecule to the V peptide, which forms a 1:1 complex in a dilute aqueous solution. In the second experimental model, the K_ds from SPR measurements reflect the adsorption of the V peptide on the monolayer of sterically hindered LA immobilized on the biosensor chip. Thus, for example, the K_ds determined from the SPR measurements do not take into account the contribution from hydrophobic interactions between the acyl chains of LA and the side chains of

nonpolar residues of the peptides, in contrast to $\Delta\Delta E_{\text{comp}}$. Nevertheless, the empirically measured K_ds and the computed $\Delta\Delta E_{\text{comp}}$ represent complementary data (with limited correlation), and both the measured and the computed data confirmed that the cationic peptides designed exhibit a strong affinity for *E. coli* LA that is comparable to the affinity of the reference antiendotoxin agent, PmB (27, 30).

The flexible V peptides underwent conformational transitions and attained different secondary structures in the vicinity of the heterogeneous membrane-mimicking interface upon association with the amphipathic LA or phosphatidylcholine molecules. Similar observations were made for other linear and cyclic peptides (37). Molecular modeling suggested that in the complexes with LA, the peptide backbone acquired an amphipathic β-hairpin structure with distinct polar and nonpolar faces induced by the amphipathic LA molecule (Fig. 6). The presence of a central hinge of the type GIG, similar to the GSG loop of each of the V peptides, was found to be responsible for the effective antibiotic activity of a composite 20-residue synthetic peptide, cecropin A (residues 1 to 8)–magainin 2 (residues 1 to 12), with a helix-hinge-helix structure (28), indicating that structural flexibility is important.

Antimicrobial activity. The MICs of five antibiotics for *E. coli*, *Klebsiella pneumoniae*, and *P. aeruginosa* were reported by Lancini et al. (15) to be 85 μg/ml for erythromycin, 6 μg/ml for tetracycline, 3 μg/ml for chloramphenicol, 1 μg/ml for rifampin, and 0.17 μg/ml for gentamicin. In comparison, the most potent peptide, V4, with an MIC of 10 ng/ml, displayed 17- to 8,500-fold greater potencies than the five established antibiotics with activities against these three bacterial species. Muhle and Tam (19) designed amphipathic cyclic cationic antimicrobial peptides similar to the V peptides with sequences such as c(PA**C**R**C**R**A**G-PA**R**C**R**C**AG), where "c" means cyclic, constrained by two cross-linking disulfide bonds. The MICs of these peptides were 20 nM for *E. coli*, which is comparable to that of V4. Also, bactericidal and permeability increasing protein, a 60-kDa LPS-binding protein, had a low MIC (less than 1 nM) for gram-negative bacteria (34). V4, with a molecular mass of 2 kDa, achieved a comparably strong antimicrobial effect.

The V peptides with the highest affinities to the *E. coli* LA did not necessarily show the strongest antimicrobial effects. This indicates that initial binding to the outer membranes of gram-negative bacteria, which differ in their LPS compositions, alone does not determine their overall antimicrobial effects. This observation is opposite earlier assumptions that strong initial binding of cationic amphipathic peptides to outer membrane components may interfere with the antimicrobial activity (21, 38). In fact, a higher affinity to the outer bacterial membrane seems to be a favorable prerequisite for the antimicrobial effects, since the V peptides displayed low micromolar K_ds to the LA of *E. coli* and antimicrobial activities at concentrations in the nanomolar range.

QSAR analysis. The validity of the QSAR model for the antimicrobial potencies of the V peptides against gram-negative bacteria was verified with the set of cyclic cationic amphipathic peptides designed by Muhle and Tam (19). These peptides displayed potent activities against gram-negative bacteria (*E. coli* and *P. aeruginosa*), with the lowest MIC starting at 160 nM. Our correlation equation for the MIC was able to repro-

duce the qualitative rank order of antimicrobial potencies at low salt concentrations for eight of nine peptides: R6F > R6A > R5Y ≅ R5W > R4A ≅ R4Y > K4A > K5L. Our correlation equation failed to correctly rank only one peptide, R5L (19).

Strategy for dissociation of antimicrobial and hemolytic effects. The QSAR correlation equations obtained for the V1 to V7 peptides permit quantitative prediction of the biological activities of the analogs of the V peptides synthesized and tested and can help elucidate whether site-directed replacements of residues in the polar and nonpolar faces of the peptides (while preserving the molecular charge and overall symmetry of the LPS- and LA-binding motifs) may lead to independent variations in their antimicrobial and hemolytic potencies.

The QSAR equation (equation 1) predicts rapid increases in the MICs with an increase in the molecular charge Q_M over 4 è when amphipathicity and hydrophobicity are kept constant at the levels of the most promising peptide, V4. Model analogs of V4 that shared the polar *HKHQHKH* motif and that differed only in the *H* residues (which retain the amphipathicity index of V4) are predicted to possess decreasing hemolytic activities with decreasing lipophilicities, while their predicted antimicrobial and cytotoxic activities remain basically unchanged. In other analogs of V4, the replacement of the two central Gln residues by more polar Asn residues is predicted to lead to significantly increased antimicrobial potencies due to the increased amphipathicity independent of the *H* residues (the predicted MICs are lower than that of V4).

Thus, the variations in the *H* residues forming the hydrophobic face of the analogs of V4 mainly affected the hemolytic activity, which was shown to depend strongly on the $\Pi_{o/w}$, but did not affect the predicted MICs∗ of the analogs. Since the hemolytic activity correlates strongly with the $\Pi_{o/w}$s of V peptides and is less dependent on Q_M and AI, we may conclude that the lysis of human erythrocytes is probably caused by the enhanced penetration of more hydrophobic peptides into the phospholipid membranes of eukaryotic cells. Therefore, replacement of the *H* residues with less hydrophobic residues in the nonpolar face of the amphipathic analogs (with the polar face kept unchanged) seems to be a suitable design strategy to reduce the hemolytic activities of the V peptides while preserving their antimicrobial potencies.

On the other hand, directed substitutions of the **B** and *P* residues in the polar faces of the V peptides with more polar residues, which increase the amphipathic character (more negative AI values) of the peptide while preserving the net charge, the symmetry of the binding motifs, and the composition of the hydrophobic face, are predicted to bring about a significant increase in antimicrobial potencies. Thus, the rational strategy of residue substitution directed to either the polar or the nonpolar faces of the cyclic V peptides might result in the dissociation of the antimicrobial and hemolytic effects of cationic amphipathic V peptides.

Implications for hypothetical mechanism of antimicrobial effect. The amphipathicities of molecules have been related to their abilities to form aggregates and supramolecular complexes (12). Increased aggregation or the formation of assemblies containing amphipathic particles on the surfaces of bacterial membranes may therefore be responsible for their

antimicrobial effects (11, 20). The aggregation of cationic peptides alone is less probable at neutral pH. However, the introduction of anionic amphipathic LA molecules into such assemblies may facilitate the formation of aggregates by cationic peptides. Recently, it was shown that PmB is able to sequester LA from the surfaces of liposomes containing dimyristoylphosphatidylethanolamine (33). Since the antimicrobial activities of the V peptides strongly increase with the increasing amphipathicities of the molecules at constant Q_M and $\Pi_{o/w}$, we hypothesize that aggregates of V peptides rather than individual molecules exert strong antimicrobial effects. The presence of antibacterial activities at concentrations in the low nanomolar range suggests that the V peptides possess an intricate mechanism of interaction with the bacterial membrane rather than a nonspecific mechanism of membrane disruption. In fact, it was reported (17) that antimicrobial peptides like magainin 2 and PGLa form peptide-lipid heterosupramolecular pores in the phospholipid bilayers, which explains the observed synergism in their antimicrobial effects. Membrane pores formed by α-helical magainin as well as β-hairpin-shaped protegrin 1 produced a diffraction pattern similar to that of the well-established transmembrane gramicidin channel (8, 10, 39). Similar conclusions which relate the ability of cationic peptides to form aggregates to their antimicrobial potencies have recently been presented for dermaseptin S4 (4), protegrin 1 (26), and human defensins (29).

The results of the present study allow us to propose that amphipathicity and, possibly, also the formation of pore-like aggregates involving the peptides and LA may by responsible for the strong antimicrobial potencies of the cationic V peptides. The general sequence Ac-C-*HBHB(P)HBH*-GSG-*HBHB(P)HBH*-C-NH$_2$ represents an almost ideal amphipathic pattern for a cyclic β-hairpin peptide. It is therefore not surprising that the MICs of the most active V peptides are about 2 orders of magnitude lower than those of known cationic antimicrobial peptides. In conclusion, the V peptides designed de novo harbor potencies unsurpassed by any known antibiotics of metabolite or peptide origin. The general sequence pattern Ac-C-*HBHB(P)HBH*-GSG-*HBHB(P)HBH*-C-NH$_2$ may be adopted for the further rational design of a repertoire of antiendotoxin peptides from which candidates with potent antimicrobial activities with high SIs may be selected through empirical tests.

REFERENCES

1. **Boman, H. G.** 1995. Peptide antibiotics and their role in innate immunity. Annu. Rev. Immunol. **13:**61–92.
2. **Dimarcq, J.-L., P. Bulet, C. Hetru, and J. Hoffmann.** 1998. Cysteine-rich antimicrobial peptides in invertebrates. Biopolymers **47:**465–477.
3. **Fauchère, J.-L.** 1996. Lipophilicity in peptide chemistry and peptide drug design, p. 355–373. *In* V. Pliška, B. Testa, and H. van der Waterbeemd (ed.), Lipophilicity in drug action and toxicity. VCH Publishers, Weinheim, Germany.
4. **Feder, R., A. Dagan, and A. Mor.** 2000. Structure-activity relationship study

of antimicrobial dermaseptin S4 showing the consequences of peptide oligomerization on selective cytotoxicity. J. Biol. Chem. **275:**4230–4238.

5. **Frecer, V., B. Ho, and J. L. Ding.** 2000. Interpretation of biological activity data of bacterial endotoxins by simple molecular models of mechanism of action. Eur. J. Biochem. **267:**837–852.

6. **Gough, M., R. E. W. Hancock, and N. M. Kelly.** 1996. Antiendotoxin activity of cationic peptide antimicrobial agents. Infect. Immun. **64:**4922–4927.

7. **Hancock, R. E. W.** 1999. Host defence (cationic) peptides: what is their future clinical potential? Drugs **57:**469–473.

8. **He, K., S. J. Ludtke, D. L. Worcester, and H. W. Huang.** 1995. Antimicrobial peptide pores in membranes detected by neutron in-plane scattering. Biochemistry **34:**15614–15618.

9. **Hoess, A., S. Watson, G. R. Siber, and R. Liddington.** 1993. Crystal structure of an endotoxin-neutralizing protein from horseshoe crab, Limulus anti-LPS factor, at 1.5 Å resolution. EMBO J. **12:**3351–3356.

10. **Huang, H. W.** 2000. Action of antimicrobial peptides: two-state model. Biochemistry **39:**8347–8352.

11. **Hwang, P. M., and H. J. Vogel.** 1998. Structure-function relationships of antimicrobial peptides. Biochem. Cell. Biol. **76:**235–246.

12. **Israelachvili, J. N., S. Marcelja, and R. G. Horn.** 1980. Physical principles of membrane organization. Q. Rev. Biophys. **13:**121–200.

13. **Kondejewski, L. H., S. W. Farmer, D. S. Wishart, C. M. Kay, R. E. W. Hancock, and R. S. Hodges.** 1996. Modulation of structure and antibacterial and haemolytic activity by ring size in cyclic gramicidin S analogs. J. Biol. Chem. **271:**25261–25268.

14. **Kondejewski, L. H., M. Jelokhani-Niaraki, S. W. Farmer, B. Lix, C. M. Kay, B. D. Sykes, R. E. W. Hancock, and R. S. Hodges.** 1999. Dissociation of antimicrobial and haemolytic activities in cyclic peptide diastereomers by systematic alterations in amphipathicity. J. Biol. Chem. **274:**13181–13192.

15. **Lancini, G., F. Parenti, and G. G. Gallo.** 1995. Antibiotics: a multidisciplinary approach, p. 19. Plenum Press, New York, N.Y.

16. **Maple, J. R., M.-J. Hwang, T. P. Stockfish, U. Dinur, M. Waldman, C. S. Ewing, and A. T. Hagler.** 1994. Derivation of class II force fields. I. Methodology and quantum force field for the alkyl functional group and alkane molecules. J. Comput. Chem. **15:**162–182.

17. **Matsuzaki, K., Y. Mitani, K. Akada, O. Murase, S. Yoneyama, M. Zasloff, and K. Miyajima.** 1998. Mechanism of synergism between antimicrobial peptides magainin 2 and PGLa. Biochemistry **37:**15144–15153.

18. **Matsuzaki, K., K. Sigishita, and K. Miyajima.** 1999. Interactions of an antimicrobial peptide, magainin 2, with lipopolysaccharide-containing liposomes as a model for outer membranes of gram-negative bacteria. FEBS Lett. **449:**221–224.

19. **Muhle, S. A., and J. P. Tam.** 2001. Design of gram-negative selective antimicrobial peptides. Biochemistry **40:**5777–5785.

20. **Oren, Z., and Y. Shai.** 1998. Mode of action of linear amphipathic α-helical antimicrobial peptides. Biopolymers **47:**451–463.

21. **Oren, Z., and Y. Shai.** 2000. Cyclization of a cytolytic amphipathic α-helical peptide and its diastereomer: effect on structure, interaction with model membranes, and biological function. Biochemistry **39:**6103–6114.

22. **Parillo, J. E.** 1993. Pathogenic mechanisms of septic shock. N. Engl. J. Med. **328:**1471–1477.

23. **Pristovšek, P., and J. Kidrič.** 1999. Solution structure of polymyxins B and E

and effect of binding to lipopolysaccharide: an NMR and molecular modelling study. J. Med. Chem. **42:**4604–4613.

24. **Ried, C., C. Wahl, T. Miethke, G. Wellnhofer, C. Landgraf, J. Schneider-Mergener, and A. Hoess.** 1996. High affinity endotoxin-binding and neutralizing peptides based on crystal structure of a recombinant Limulus anti-lipopolysaccharide factor. J. Biol. Chem. **271:**28120–28127.

25. **Rost, B., and C. Sander.** 1994. Combining evolutionary information and neural networks to predict protein secondary structure. Proteins **19:**55–72.

26. **Roumestand, C., V. Louis, A. Aumelas, G. Grassy, B. Calas, and A. Chavanieu.** 1998. Oligomerization of protegrin-1 in the presence of DPC micelles. A proton high-resolution NMR study. FEBS Lett. **421:**263–267.

27. **Rustici, A., M. Velucchi, R. Faggioni, M. Sironi, P. Ghezzi, S. Quataet, B. Green, and M. Porro.** 1993. Molecular mapping and detoxification of the lipid A binding site by synthetic peptides. Science **259:**361–365.

28. **Shin, S. Y., J. H. Kang, S. Y. Jang, Y. Kim, K. L. Kim, and K.-S. Hahm.** 2000. Effects of the hinge region of cecropin A(1–8)-magainin 2(1–12), a synthetic antimicrobial peptide, on liposomes, bacterial and tumor cells. Biochim. Biophys. Acta **1463:**209–218.

29. **Skalicky, J. J., M. E. Selsted, and A. Pardi.** 1994. Structure and dynamics of the neutrophil defensins NP-2, NP-5, and HNP-1: NMR studies of amide hydrogen exchange kinetics. Proteins **20:**52–67.

30. **Srimal, S., N. Surolia, S. Balasubramanian, and A. Surolia.** 1996. Titration calorimetric studies to elucidate the specificity of the interactions of polymyxin B with lipopolysaccharides and lipid A. Biochem. J. **315:**679–686.

31. **Takada, H., and S. Kotani.** 1989. Structural requirements of lipid A for endotoxic and other biological activities. Crit. Rev. Microbiol. **16:**477–523.

32. **Tan, N. S., P. M. L. Ng, Y. H. Yau, P. K. Chong, B. Ho, and J. L. Ding.** 2000. Definition of endotoxin binding sites in horseshoe crab factor C recombinant sushi proteins and neutralization of endotoxin by sushi peptides. FASEB J. **14:**1801–1813.

33. **Thomas, C. J., N. Surolia, and A. Surolia.** 1999. Surface plasmon resonance studies resolve the enigmatic endotoxin neutralizing activity of polymyxin B. J. Biol. Chem. **274:**29624–29627.

34. **Tobias, P. S., K. Soldau, N. M. Iovine, P. Elsbach, and J. Weiss.** 1997. Lipopolysaccharide (LPS)-binding proteins BPI and LBP form different types of complexes with LPS. J. Biol. Chem. **272:**18682–18685.

35. **Tracey, K. J., Y. Fong, D. Hesse, K. R. Manogue, A. T. Lee, G. C. Kuo, S. F. Lowry, and A. Cerami.** 1987. Anti-cachectin/TNF monoclonal antibodies prevent septic shock during lethal bacteraemia. Nature **330:**662–664.

36. **Wiedeman, B., and H. Grimm.** 1996. Susceptibility to antibiotics: species incidence and trends, p. 900–1168. In V. Lorian (ed.), Antibiotics in laboratory medicine, 4th ed. The Williams & Wilkins Co., Baltimore, Md.

37. **Woody, R. W.** 1995. Circular dichroism. Methods Enzymol. **246:**34–71.

38. **Wu, M., and R. W. E. Hancock.** 1999. Interaction of the cyclic antimicrobial cationic peptide bactenectin with the outer and cytoplasmic membrane. J. Biol. Chem. **274:**29–35.

39. **Yang, L., T. M. Weiss, T. A. Harroun, W. T. Heller, and H. W. Huang.** 1998. Neutron off-plane scattering of aligned membranes. I. Method of measurement. Biophys. J. **75:**641–645.

40. **Yau, Y. H., B. Ho, N. S. Tan, P. M. L. Ng, and J. L. Ding.** 2001. High therapeutic index of factor C sushi peptides: potent antimicrobials against Pseudomonas aeruginosa. Antimicrob. Agents Chemother. **45:**2820–2825.